Video Article

Intravenous Injections in Neonatal Mice

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URL: https://www.jove.com/video/52037
DOI: doi:10.3791/52037

Keywords: Basic Protocol, Issue 93, intravenous injection, systemic delivery, neonate, AAV, gene therapy, brain, spinal cord, muscle, temporal vein

Date Published: 11/11/2014

Citation: Gombash Lampe, S.E., Kaspar, B.K., Foust, K.D. Intravenous Injections in Neonatal Mice. *J. Vis. Exp.* (93), e52037, doi:10.3791/52037 (2014).

Abstract

Intravenous injection is a clinically applicable manner to deliver therapeutics. For adult rodents and larger animals, intravenous injections are technically feasible and routine. However, some mouse models can have early onset of disease with a rapid progression that makes administration of potential therapies difficult. The temporal (or facial) vein is just anterior to the ear bud in mice and is clearly visible for the first two days after birth on either side of the head using a dissecting microscope. During this window, the temporal vein can be injected with volumes up to 50 µl. The injection is safe and well tolerated by both the pups and the dams. A typical injection procedure is completed within 1-2 min, after which the pup is returned to the home cage. By the third postnatal day the vein is difficult to visualize and the injection procedure becomes technically unreliable. This technique has been used for delivery of adeno-associated virus (AAV) vectors, which in turn can provide almost body-wide, stable transgene expression for the life of the animal depending on the viral serotype chosen.

Video Link

The video component of this article can be found at https://www.jove.com/video/52037/

Introduction

Delivery of therapeutics to the central nervous system (CNS) in murine models of pediatric disease remains a challenge. Mice that model newborn disease states are undersized and developmentally immature, and therefore can be difficult to directly inject in appropriate structures within the CNS. Intravascular injection of therapeutic agents is a non-invasive, well tolerated method to deliver cells, drugs, or viral vectors to the entire body including the CNS[1-5] and retina[3,5-9]. Previous publications describe temporal face vein injection using a transilluminator[10,11], without a dissection microscope[11,12], or requiring two individuals to inject[10]. The injection technique described in this protocol is advantageous because a single individual can inject pups, and the light source to view the temporal vein is not touching the pup, eliminating the need for surgical tape or the attachment of a pup to a fixed surface such as a transilluminator[11]. Delivery of adeno-associated viral vector serotype 9 (AAV9) in mice produces robust expression in neurons and astrocytes throughout the brain and spinal cord (**Figure 1**). Intravascular delivery of viral vectors into the superficial temporal facial vein has been reliably used in various studies in neonatal mice to treat the pediatric neuromuscular disorder Spinal Muscular Atrophy (SMA)[2,4,13,14] and ultimately increased the lifespan of treated mice.

Intravascular injection of neonatal mice also effectively targets the peripheral nervous system and peripheral organs (**Figure 2**). Following injection of AAV, transduction of dorsal root ganglia, liver, heart, skeletal muscle, lung, and myenteric plexus of the gut has been observed[1,3,6,7,15]. Widespread transduction of the CNS and periphery makes this method of injection ideal for diseases requiring global expression of a transgene, such as Gaucher's disease[16] and other lysosomal storage diseases[17,18], Batten's disease and related neuronal ceroid lipofuscinoses,[19] and Bardet-Biedl syndrome, a genetic multisystem disorder with onset of symptoms occurring in early childhood[20]. Intravascular injection into neonatal mice should also be considered as a novel method of modeling system-wide pediatric diseases. This technique has been translated to larger animal models[5,21] and intravascular injection already exists as a clinically acceptable method of delivering therapeutics.

The current protocol describes a simple, efficient method of delivering agents to neonatal mice through the superficial temporal face vein no later than postnatal day 2. Injection can be completed by a single, practiced individual and is well tolerated by both the pups and the dams. Pups experience minimal distress and recover quickly. Importantly, successful injection will result in global delivery of the agent administered. This protocol is appropriate for delivery of viral vectors, pharmaceutical agents or cells to newborn mice.

Protocol

All procedures listed in the protocol have been approved Institute for Animal Use and Care committee (IACUC) of the Ohio State University.

1. Preparation of Workspace

1. Gather wet ice to anesthetize the mouse pups, an empty cage to segregate the dam from the litter, a dissecting microscope, a light source that can be positioned at an angle to the injection (use of a light source at a 90° angle to the injection site obscures the vein), a clean surface to place the animal for the injection, cotton swabs, 3/10 cc insulin syringe with 3/8" 30 G needle (one per animal) and 1% Evans Blue Dye (made with phosphate buffered saline (PBS)) solution for training.
2. Remove the dam to a separate cage while manipulating the pups.

2. Injection Procedure

1. Place a single pup directly on the wet ice for 30-60 sec to anesthetize the animal. Do not leave the animal on ice too long due to a risk of hypothermia related complications including ventricular fibrillation, tissue hypoxia and metabolic acidosis.
   NOTE: Our experience is that 30-60 sec is sufficient to slow the pup movements to allow for injection. If deeper anesthesia is required, inhalants such as 1-2% isoflurane may be appropriate.
2. While the animal is on ice, load the syringe with 30 µl of Evans blue dye.
3. When the animal is fully anesthetized, confirmed by lack of movement on the ice while still breathing, move it under the microscope. For a right-handed injection, face the animal's muzzle to the right. Place the left index finger on the muzzle and the left middle finger caudal to the ear bud so that the ear bud is between the index and middle fingers (**Figure 1**).
4. Examine just anterior to the ear bud for a superficial capillary that moves when the skin is manipulated. This capillary is NOT the target, however identification is important for temporal vein identification. Next, locate a dark, shadowy vein inferior to the capillary that remains fixed regardless of skin position. The temporal vein appears shadowy, runs dorsal to ventral, and feeds into the jugular vein (**Figure 1**).
5. Enter the temporal vein with the needle bevel up. If correctly inserted, it is possible to view the needle bevel fill with blood through the skin. Then depress the plunger slowly and note blanching of the vein down the side of the face.
6. Allow the needle to remain within the vein for an added 10-15 sec to prevent backflow of the injectant.

3. Post-injection

1. After a proper injection, the pup should turn blue almost immediately. Remove the needle and use gentle force to apply a cotton swab to the injection site until the blood clots.
2. Monitor the pup for signs of distress. Allow the pup 2-3 min to recover and rewarm, recognize when the pup is conscious, upright and moving, before returning to the cage. Cup the pups in the investigator's gloved hands to provide appropriate warmth to aid in recovery if necessary. Alternatively, place a heating pad under the home cage to facilitate warming the injected pup. Generally after an Evans blue dye injection, euthanize pups. Do not include dye when injecting test material.
3. Place the pup back into the home cage and ensure the pup is coated with bedding and/or nestlet to ensure reacceptance by the dam.
4. Use a new syringe and cotton swab for each pup to maintain sterility.

Representative Results

During a proper injection, the vein should momentarily turn clear, or blanch. If injecting dye the entire pup should turn blue within seconds. If an improper injection has occurred, there is often a concentrated subcutaneous bolus in the head or neck and injectant may leak out of the injection site. Improper injections may also result in the appearance of bruising around the throat. Pups that receive subcutaneous injections (*i.e.* the injection was not fully delivered in the vein) generally experience no ill side effects and routinely respond to treatment as the properly injected mice do.

Depending on the therapy delivered, successful injection can result in widespread delivery to the CNS and peripheral organs. When injecting self complimentary adeno-associated virus serotype 9 expressing green florescent protein (GFP) with a chicken β-actin hybrid promoter (CB) (together referred to as "scAAV9 CB GFP"), transgene expression is found in glial cells and neurons across multiple brain regions and within the spinal cord (**Figures 2A-2C**). Intravascular injection of scAAV9 CB GFP also results in widespread expression throughout the periphery, with noticeable expression in heart and liver tissue (**Figures 3A-3D**).

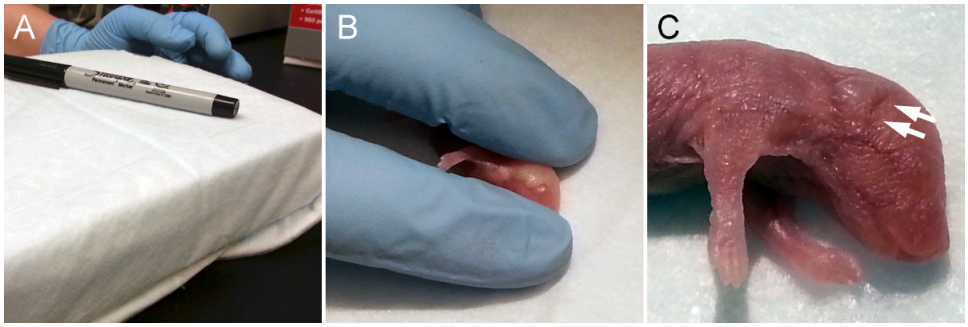

Figure 1: (A) A makeshift injection platform made from an absorbant pad ("diaper") wrapped around a Styrofoam rack from 50 ml conical packaging. The platform allows the investigator to rest their hand on the table adjacent to the platform. This allows the investigator to perform the injection at a flatter angle relative to the mouse pup. **(B)** Image showing pup immobilization between the fore and middle fingers. **(C)** Image showing a P2 pup after cryoanesthesia. The vein for injection is indicated by the white arrows. Please click here to view a larger version of this figure.

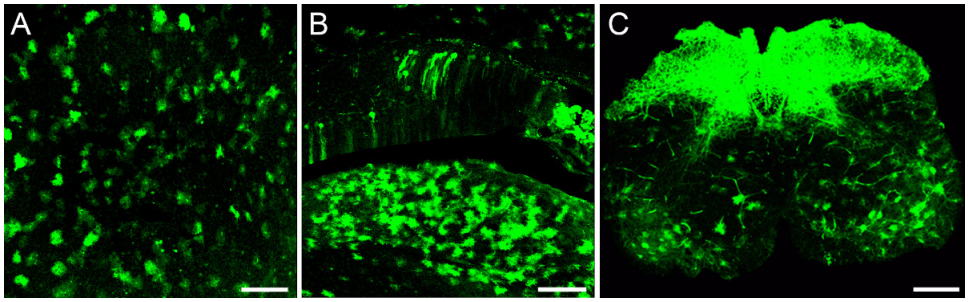

Figure 2: Central nervous system gene expression following neonate intravenous injection of self complementary AAV9 expressing green fluorescent protein (scAAV9 CB GFP). Postnatal day 1 (P1) mouse pups were intravenously injected with 1 x 10^{11} vector genomes (vg) of self-complementary (sc) scAAV9 CB GFP. Four weeks post injection, mice were sacrificed and perfused with 4% paraformaldehyde. Tissues were collected, sliced and stained for GFP expression. GFP expression is seen throughout the brain including the anterior striatum **(A)** through the pons and cerebellum **(B)**. GFP positive cells are typically neurons and astrocytes. GFP expression is also detected in the spinal cord **(C)**. Spinal motor neurons and dorsal root ganglia neurons are efficiently transduced using neonate IV delivery. GFP positive vasculature and astrocytes are also seen throughout the spinal grey matter. All scale bars = 200 µm. Please click here to view a larger version of this figure.

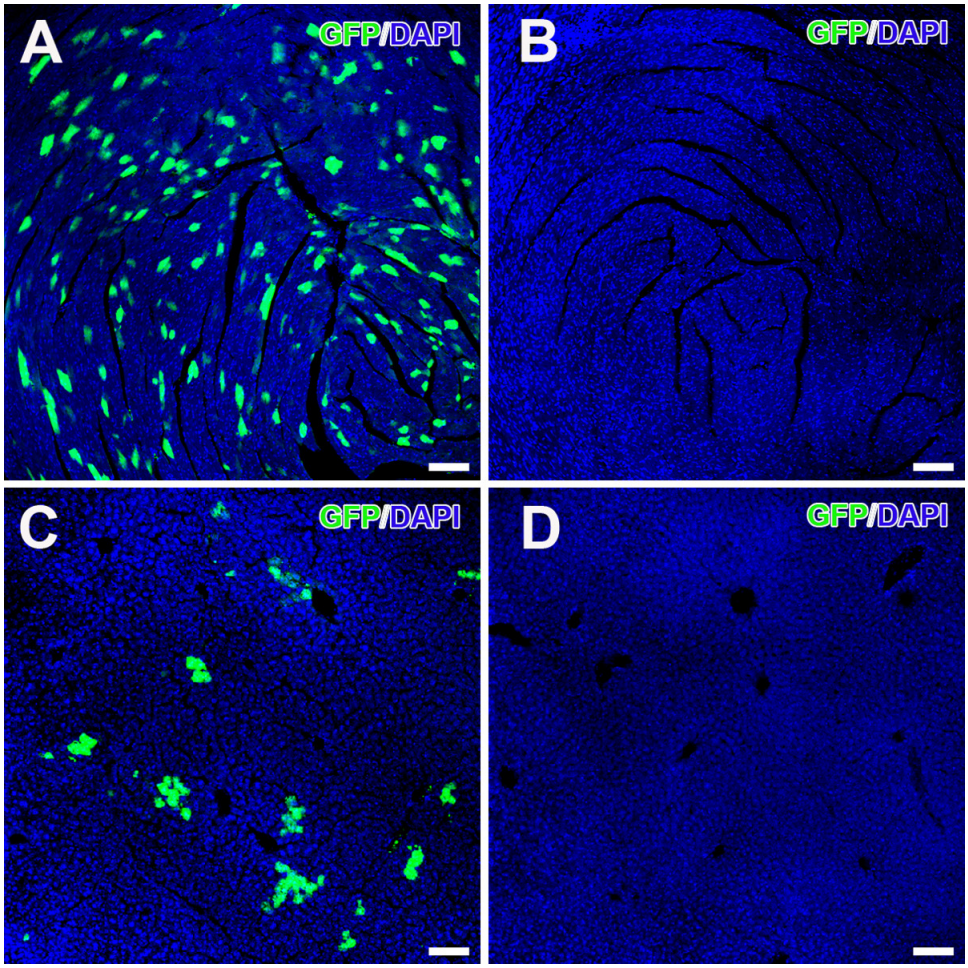

Figure 3: GFP expression in peripheral organs following neonatal intravenous injection of self complementary AAV9 GFP (scAAV9 CB GFP). GFP immunofluorescence is detectable in the adult mouse heart **(A)** and liver **(C)** following neonatal intravenous injection of scAAV9 CB GFP. For comparison, GFP expression is absent in the heart **(B)** and liver **(D)** of uninjected mice. All scale bars = 100 µm.

Discussion

Intravascular delivery of agents to the CNS or throughout the body is difficult in neonatal murine models of disease. The described protocol is a quick, relatively non-invasive way to intravenously administer solutions into neonatal mice with minimal equipment requirements. Though the temporal face vein can be viewed by the naked eye, injections may have greater accuracy with the use of the microscope and fiber-optic light source, especially for an unpracticed injector. Intravascular injections in neonatal mice have a high success rate[12], and AAV transduction has been observed in early pups even if part of the injectant is administered subcutaneously. The injection is well tolerated by the mouse pups, and dams on FVB/N or C57BL/6 backgrounds tolerate manipulation of the pups very well. However, in rare instances dams can destroy entire litters. These injections can be successfully performed in mice as small as 0.8 g through 1.6 g. Alternative injection routes for neonate mice have been published by us and others including retro-orbital, intrajugular and intraperitoneal[10,15,22]. We prefer the injection route described here because it is clinically relevant, able to be performed by a single experimenter with no special equipment requirements and allows for a wide range of volumes to be administered.

A common problem experienced when performing injections is difficulty viewing the temporal vein and therefore misplacement of the needle. The fiber-optic light source should be positioned at <90° and shining downward on the head when being held for best viewing of the vein. Injectors should take time to adjust the light source to suit their needs. Temporal vein observation is significantly decreased if pups are too large, characterized as older then P2 or greater than 2.0 g. A second problem may be determining if the needle bevel has entered the vein. The temporal facial vein is superficial, so care must be taken to avoid entering beneath the vein. Ensuring that the needle bevel is entering parallel to the vein will prevent puncturing through the vein. Needle placement is best verified by slow injection and subsequent blanching of the vein.

The superficial temporal vein is visible on either side of the head. Left-handed individuals may find that facing the animal to the investigator's left may be ergonomically better and should be determined for each individual investigator. Should the vein not be visible or is mistargeted on initial attempts, targeting the vein on the opposite side of the head is appropriate. If the vein is punctured on both sides of the head, some leakage may occur through the first site during injection into the second site. Depending on the working distance of the microscope, it may be appropriate to build a small platform on which to inject the animal; for example, an empty Styrofoam container from 50 ml conical tube packaging (**Figure 1**). This allows the investigator to position the animal near the edge of the platform so that the investigator's injecting hand can rest directly on the table. This can improve the angle of entry into the vein and minimize light obstruction.

There are a few limitations to the utility of the protocol including the injection volume, the age window for injections and the unpredictability of the dam response. Injection volumes for pups are limited to 50 µl. We have previously dosed animals with 100 µl, but have observed increased injection related mortality compared to lower volume injections, but others report successfully delivering 100 µl[10,12]. Further, it has been suggested that increased injection volumes can damage the blood brain barrier[23]. Often the experimental reagent is mixed with PBS to maintain a constant 50 µl injection volume. We are unaware of studies examining the effect of injection volume on spread throughout the neonate mice, and empirical studies should be performed to identify effects on injection volume for new reagents at a given dose. A second limitation is that the superficial temporal vein is easily visible on the first and second postnatal days. Changes to the animal size, skin pigmentation and thickness combine to obscure the vein at later ages though others report accessing the vein through postnatal day 6[10,12]. Alternatively, we used ultrasound guided cardiac injections to deliver viral vector to the circulation in P5 and P10 animals[4]. Finally, the acceptance of the mouse dams of the procedure is unpredictable. We have treated FVB/N, C57BL/6 and B6SJL mice across mouse models of neuromuscular disease and autism spectrum disorders. FVB/N mice are the most tolerant while strains on the C57BL/6 background can occasionally destroy individual pups or entire litters, but this a rare occurrence. However, this should be a consideration when planning for sufficient *n* for an experiment. Dam acceptance can be further influenced by what disease the mouse strain is modeling. For example, a model of an autism spectrum disorder had a much higher rate of pup rejection than expected based on the background strain. Fostering pups may be a consideration if dam acceptance is a concern. Other approaches to limit rejection include performing injections in a separate room from the dam to limit access to pup ultrasonic vocalizations, and to rub the dam's nose and/or the pups with a cloth dampened with 70% ethanol to mask any investigator scent. Pups should also be rubbed with dirty bedding to aid in acceptance.

This protocol has been successfully used to treat a mouse model of SMA. SMA is a pediatric neurodegenerative disease that primarily affects lower motor neurons of the spinal cord. Without treatment, this mouse model dies by 15 days after birth on average. After intravenous injections of AAV gene therapy in 1 day-old mice, survival was extended to one year, demonstrating the safety and tolerability of the described injection protocol[2,4,14]. Similar results were also obtained in the same SMA mouse model following IV injection of antisense oligonucleotides[8,9]. Future applications include the delivery of other cell and gene therapies, as well as the creation of neonatal models for developmental disorders of the CNS and the periphery to exogenously express a cDNA or an RNAi cassette[24]. Intravenous injection is an approved clinical method of delivery; therefore, utilization of this technique may be advantageous in preclinical studies for a variety of agents.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors wish to acknowledge the NINDS, FightSMA, and Families of SMA for financial support. SEGL is supported by NINDS training grant #5T32NS077984-02.

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